Advances in Medical Sciences 65 (2020) 348–353

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original research article

Quantitative changes in selected bacteria in the stool during the treatment of Crohn's disease

Dominika Salamon[a], Tomasz Gosiewski[a], Agnieszka Krawczyk[a], Agnieszka Sroka-Oleksiak[a], Mariusz Duplaga[b], Krzysztof Fyderek[c], Kinga Kowalska-Duplaga[c,*]

[a] Jagiellonian University Medical College, Faculty of Medicine, Chair of Microbiology, Department of Molecular Medical Microbiology, Krakow, Poland
[b] Jagiellonian University Medical College, Faculty of Health Sciences, Institute of Public Health, Department of Health Promotion, Krakow, Poland
[c] Jagiellonian University Medical College, Faculty of Medicine, Department of Pediatrics, Gastroenterology and Nutrition, Krakow, Poland

ARTICLE INFO

Keywords:
Children
Inflammatory bowel disease
Crohn's disease treatment
Gut microbiome
qPCR

ABSTRACT

Purpose: The aim of this study was to determine quantitative changes in selected species of bacteria (*Bacteroides fragilis, Lactobacillus fermentum, Lactobacillus rhamnosus, Serratia marcescens*) in the stool of patients with Crohn's disease (CD) in the course of induction treatment with exclusive enteral nutrition (EEN) or anti-tumor necrosis factor alpha (Infliximab, IFX) vs. healthy controls (HC).

Materials/methods: DNA was isolated from stool samples of CD (n = 122) and HC (n = 17), and quantitative real-time Polymerase Chain Reaction (qPCR) was applied. In both treatment groups, the first stool sample was taken before the start of treatment, and the second 4 weeks after its end: in EEN (n = 48; age (mean; SD) 13.35 ± 3.09 years) and IFX groups (n = 13; age (mean; SD) 13.09 ± 3.76 years).

Results: The only species that showed a statistically significant difference between the two groups of patients before any therapeutic intervention was *L. fermentum*. Moreover, its number increased after completion of EEN and differed significantly when compared with the HC. In the IFX group the number of *L. fermentum* decreased during the therapy but was significantly higher than in the HC. The number of *S. marcescens* in the EEN group was significantly lower than in the controls both before and after EEN.

Conclusion: The implemented treatment (EEN or IFX) modifies the microbiome in CD patients, but does not make it become the same as in HC.

1. Introduction

Over the past few years, there has been an increase in incidence of inflammatory bowel disease (IBD), including Crohn's disease (CD), in younger age groups all over the world, particularly in newly industrialized European countries, now following the so-called Western pattern diet [1,2].

The IBD etiology is extremely complex and still not fully explained. The intestinal microbiome, especially the disturbance of the balance in the composition of gastrointestinal microorganisms (dysbiosis), seems to be an important mechanism in the induction and maintenance of inflammation in CD patients [3]. Numerous studies indicate the association between a reduced number of Gram-positive bacteria and, probably compensatory, increased numbers of Gram-negative bacteria and the occurrence of CD [4]. It is likely that lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is involved in the development of the disease, probably through the effect on the immune system and the stimulation of inflammation [5,6]. The better knowledge on the influence of particular species of bacteria on the course of CD could help to develop individual therapies aimed at the modification of the composition of microbial guts, and thus, the alleviation or elimination of inflammation [7].

The principles of the treatment of pediatric patients are based on the recommendations of the European Crohn's and Colitis Organisation (ECCO) and European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [8]. In newly diagnosed CD patients with mild to moderate disease activity, the exclusive enteral nutrition therapy (EEN) is the first choice of treatment. For 6–8 weeks, the patient's diet consists only of standard, liquid, polymeric formulas and is followed by pharmacological treatment with thiopurines or methotrexate (conventional therapy) and mesalazine to maintain remission [8]. Patients with high activity of CD and those who failed to

* Corresponding author. Jagiellonian University Medical College, Faculty of Medicine, Department of Pediatrics, Gastroenterology and Nutrition, Wielicka 265, 30-663, Krakow, Poland.
E-mail address: kinga.kowalska-duplaga@uj.edu.pl (K. Kowalska-Duplaga).

https://doi.org/10.1016/j.advms.2020.06.003
Received 13 November 2019; Received in revised form 3 June 2020; Accepted 12 June 2020
Available online 23 June 2020

Table 1
Primer sequences of selected species of bacteria and thermal amplification programs used in the study.

Target species	Primer sequence 5'→ 3'	Reference	Thermal amplification program
Bacteroides fragilis	(F)TCRGGAAGAAAGCTTGCT (R)CATCCTTTACCGGAATCCT	[29]	95 °C – 5 min 95 °C – 30 sec 45 °C – 30 sec } 50x 72 °C – 30 sec
Lactobacillus fermentum	(F)AACCGAGACCACCGCGTTAT (R)ACTTAACCTTACTGATCGTAGATCAGTC	[25]	95 °C – 5 min 95 °C – 30 sec 51 °C – 30 sec } 40x 72 °C – 30 sec
Lactobacillus rhamnosus	(F)CGGCTGGATCACCTCCTTT (R)GCTTGAGGGTAATCCCCTCAA	[30]	95 °C – 5 min 95 °C – 30 sec 52.4 °C – 30 sec } 50x 72 °C – 30 sec
Serratia marcescens	(F)TGCCTGGAAAGCGGCGATGG (R) CGCCAGCTCGTCGTTGTGGT	[22]	95 °C – 5 min 95 °C – 20 sec 55 °C – 30 sec } 39x 72 °C – 1 sec

respond to conventional therapy are candidates for biological therapy, e.g. with anti-tumor necrosis factor alpha. Infliximab (IFX) is used as a first-line biologic agent in pediatric CD. Doses of 5 mg/kg are given intravenously in 0-, 2- and 6-week modes as induction therapy, and then every 8 weeks as a maintenance treatment [9].

To enhance the current knowledge of the contribution of intestinal bacteria to CD development, we analyzed the relationship between the number of selected species of colorectal bacteria and the type of treatment implemented (EEN or IFX) in CD patients compared with control group. To determine the number of bacteria we used a highly specific, quantitative Polymerase Chain Reaction (qPCR) test. We also attempted to assess the association between the number of bacteria and basic biochemical parameters in patients from the studied groups. Due to the complexity of the role of microbiome in CD development and richness of the microorganisms involved, and thus the technical requirements for their identification, only 4 of the most important bacterial species were assessed in the study: 2 species of Gram-positive bacteria, as representatives of microorganisms with probiotic properties (*Lactobacillus fermentum* and *Lactobacillus rhamnosus*) [10] and 2 species of Gram-negative bacteria, commonly occurring in the environment, and under certain conditions, considered potentially pathogenic to humans (*Bacteroides fragilis* and *Serratia marcescens*) [11,12].

2. Material and methods

Children from 2 to 18 years of age, diagnosed with CD according to the revised Porto criteria [13], hospitalized at the Department of Pediatrics, Gastroenterology and Nutrition, University Children's Hospital in Krakow, Poland, between the years 2015-2018, were recruited into 2 groups. The first group consisted of 48 patients with newly diagnosed CD who received EEN for 6 weeks as the induction of remission. In this group, we obtained the first stool sample before treatment and the second sample 3–4 weeks after completion of EEN. In the second group, there were 13 CD patients who received IFX (Remsima®, Celltrion Healthcare, Incheon, South Korea) because they failed to respond or became unresponsive to the conventional maintenance treatment with thiopurines or methotrexate. In this group, IFX was given intravenously in standard 0-, 2- and 6-week modes as induction therapy, and the first stool sample was obtained before the first dose of IFX and the second 4 weeks after the third induction dose of IFX. The control group comprised 17 healthy, unrelated children, who did not meet exclusion criteria and from whom only one stool sample was obtained.

Exclusion criteria included: age below 2 or over 18 years, treatment with antibiotics and/or probiotics 3 months before collecting fecal samples, confirmed acute or chronic gastrointestinal infections, active neoplastic disease, congenital and/or acquired immune deficiencies and lack of consent to be included in the study.

The stool samples collected into sterile containers at hospital (patients) or at home (controls) were stored under refrigerated conditions for up to 24 h, and then kept deep-frozen (−70 °C) until analysis was done. All CD patients underwent also routine hematological and biochemical testing (erythrocyte sedimentation rate, glucose, serum albumin, protein and iron concentration). The blood samples were taken at the same time points as the stool samples. The activity of the disease was evaluated by using Pediatric Crohn's Disease Activity Index (PCDAI; maximal score: 100 points; 0–10 points – inactive disease, 10–30 points – mild disease, > 30 points – moderate to severe disease).

2.1. Ethical issues

The protocol of the study was approved by Jagiellonian University Ethics Committee in Krakow, Poland (decisions numbers: 122.6120.67.2015 and 122.6120.68.2015 from 30.04.2015).All performed procedures were in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was signed by patients' parents or legal guardians (for all patients under 18 years of age) and, in addition by patients themselves, if above 16 years old.

2.2. DNA extraction from the stool samples

In this study, a modified procedure by Kowalska-Duplaga et al. [14] was used for DNA extraction. Bacterial DNA was isolated from 139 fecal samples using Genomic Mini AX Stool Spin (A&A Biotechnology, Gdansk, Poland) while applying the preliminary procedure developed by Gosiewski et al. [15]. The next steps of DNA extraction were carried out according to the A&A Biotechnology's procedure.

2.3. Quantitative real-time PCR (qPCR)

The extracted DNA was quantitatively examined for the following selected bacteria: *B. fragilis, L. fermentum*, *L. rhamnosus*, and *S. marcescens* by qPCR using the CFX96 thermocycler (BioRad, California, USA).

To detect specific DNA sequences, ready-to-use real-time PCR Mix SYBR® C (A&A Biotechnology) kit, pairs of specific primers (Genomed, Warszawa, Poland) for selected bacterial species and thermal amplification programs were used (Table 1). The number of selected microorganisms was calculated per gram of stool by interpolating the cycle threshold (Ct) values obtained from the samples relative to appropriate standard calibration curve.

2.4. Statistical analysis

Descriptive statistics were calculated for quantitative variables. The intergroup differences of continuous variables not following normal distribution were assessed with the Kruskal–Wallis test. In the case of significant differences, post-hoc test was applied. The differences between pairs of observations (before and after treatment) were evaluated with the sign test. To determine the correlation between variables, the Spearman's rank correlation coefficient (R) was calculated. The p level < 0.05 was assumed to be statistically significant. Statistical analysis was carried out with the Statistica 13.1 software (StatSoft, Inc. Tulsa, Oklahoma, USA).

3. Results

We included 61 CD patients and 17 healthy children into the study. The characteristics of the study groups are presented in Table 2.

There was a statistically significant decrease in PCDAI values after treatment in both EEN ($p < 0.001$) and IFX ($p < 0.001$) groups, which indicates a significant decrease in disease activity (Table 2). A total of 139 fecal samples ($2 \times 48 = 96$ samples in the EEN group, $2 \times 13 = 26$ in the IFX group and 17 in the control group) were quantitatively evaluated for the presence of bacterial DNA of selected bacterial species using qPCR. The results are presented in Table 3.

The number of all tested bacteria changed during the treatment. However, statistically significant differences were found only in relation to two species: *S. marcescens* and *L. fermentum*. The number of *S. marcescens* was significantly lower both before ($p < 0.001$) and after ($p < 0.001$) the EEN in comparison to the control group. The number of *L. fermentum* at baseline was statistically higher ($p = 0.001$) in the IFX group from that in the controls. The number of these bacteria was also statistically significantly lower in the patients treated with IFX at the end of induction therapy compared to both the control group ($p = 0.012$) and patients before treatment ($p = 0.013$). In the group of children treated with EEN the number of *L. fermentum* after treatment increased and was statistically significantly higher from the number in the controls ($p < 0.001$). Comparing both groups of patients (EEN *vs.* IFX), a statistically significant difference in the bacterial count was found only in the case of *L. fermentum* ($p = 0.013$) and only at the pre-treatment stage (4.55×10^9 CFU/g *vs.* 2.34×10^{10} CFU/g).

There were no statistically significant differences in the number of *B. fragilis* and *L. rhamnosus* between the investigated groups of patients and controls at any time of the study.

As for biochemical parameters, there was no statistically significant correlation, both before and after nutritional therapy between the number of selected bacterial species and glucose, protein, hemoglobin or C-reactive protein in the blood serum. Only in the IFX group, a positive correlation was observed between iron concentration and the number of *B. fragilis* both before ($R = 0.56$; $p < 0.05$) and after ($R = 0.64$; $p < 0.05$) therapeutic intervention.

4. Discussion

Numerous studies on CD etiology point to the changes of gut microbiota, although it is not clear whether the intestinal dysbiosis is the cause or the effect of inflammation in this disease [3,7,16,17]. It is

Table 2
Baseline data of the study groups.

Characteristics	EEN (n = 48)	Biological therapy – IFX (n = 13)	Control group (n = 17)
Male:Female, n (%)	29 (60%):19 (40%)	7 (54%):6 (46%)	9 (53%):8 (47%)
Age at diagnosis, years; mean (SD)	13.35 (3.09)	11.41 (4.01)	N/A
Age at initial treatment, years; mean (SD)	13.35 (3.09)	13.09 (3.76)	11.73 (SD ± 2.88) †*
Weight, kg; mean (SD)	40.93 (14.05)	41.97 (16.3)	42.8 (17.2)
Height, cm; mean (SD)	155.3 (19.1)	149.95 (20.31)	148.7 (18.80)
BMI, kg/m^2; mean (SD)	16.4 (2.92)	17.89 (3.62)	18.3 (3.80)
Pharmacological treatment			
CS, n	5	2	N/A
AZA, n	42	9	N/A
5-ASA, n	48	10	N/A
MTX, n	0	2	N/A
PCDAI-1, mean (SD)	32.03 (15.01)	47.5 (16.43)	N/A
PCDAI-2, mean (SD)	5.93 (11.36)	9.04 (6.50)	N/A
Biochemical parameters			
Glucose 1, mmol/l; mean (SD)	4.77 (0.86)	5.05 (0.88)	N/A
Glucose 2; mmol/l mean (SD)	4.62 (0.71)	5.0 (0.82)	N/A
Protein 1; g/l, mean (SD)	68.57 (7.5)	74.3 (7.39)	N/A
Protein 2; g/l, mean (SD)	75.96 (6.34)	78.65 (4.97)	N/A
Iron 1; umol/l mean (SD)	7.27 (5.7)	7.78 (3.44)	N/A
Iron 2; umol/l mean (SD)	11.96 (9.07)	8.46 (4.72)	N/A
Hgb 1, g/dl, mean (SD)	11.34 (1.91)	11.57 (1.87)	N/A
Hgb 2, g/dl, mean (SD)	12.82 (1.16)	11.99 (1.62)	N/A
CRP 1, mg/dl, mean (SD)	34.88 (35.36)	11.53 (11.41)	N/A
CRP 2, mg/dl, mean (SD)	8.13 (8.36)	8.13 (7.27)	N/A

†age at sampling
*p-value for the comparison of age in the study groups > 0.05
Abbreviations: SD – standard deviation; EEN – exclusive enteral nutrition; IFX – infliximab; CS – corticosteroids; AZA – azathioprine; 5-ASA – mesalazine; MTX – methotrexate; PCDAI-1 – Pediatric Crohn's Disease Activity Index prior to therapeutic intervention; PCDAI-2 – Pediatric Crohn's Disease Activity Index after therapeutic intervention; Glucose 1 – glucose concentration prior to therapeutic intervention; Glucose 2 - glucose concentration after therapeutic intervention; Protein 1 - protein concentration prior to therapeutic intervention; Protein 2 - protein concentration after therapeutic intervention; Iron 1 - iron concentration prior to therapeutic intervention; Iron 2 – iron concentration after therapeutic intervention; Hgb 1- hemoglobin concentration prior to therapeutic intervention; Hgb 2- hemoglobin concentration after therapeutic intervention; CRP 1 – C-reactive protein concentration prior to therapeutic intervention; CRP 2 - C-reactive protein concentration after therapeutic intervention.

Table 3
Quantitative assessment of selected bacterial species in examined fecal samples of EEN and IFX treatment groups and healthy controls.

BACTERIAL SPECIES	CONTROL mean (± SD) [CFU/g] n = 17	BEFORE EEN mean (± SD) [CFU/g] n = 48	AFTER EEN mean (± SD) [CFU/g] n = 48	p*	p**	p***	BEFORE IFX mean (± SD) [CFU/g] n = 13	AFTER IFX mean (± SD) [CFU/g] n = 13	p*	p**	p***
Bacteroides fragilis	1.45×10^9 ($\pm 5.69 \times 10^9$)	2.24×10^7 ($\pm 8.7 \times 10^7$)	4.41×10^7 ($\pm 1.8 \times 10^8$)	0.102	0.842	0.063	3.49×10^8 ($\pm 6.73 \times 10^8$)	1.68×10^7 ($\pm 3.18 \times 10^7$)	0.102	0.842	0.382
Lactobacillus fermentum	3.31×10^8 ($\pm 4.95 \times 10^8$)	$4,55 \times 10^9$ ($\pm 1.24 \times 10^{10}$)	1.2×10^{10} ($\pm 3.26 \times 10^{10}$)	1	**0.00001**	0.285	2.34×10^{10} ($\pm 3.53 \times 10^{10}$)	$4,76 \times 10^9$ ($\pm 6.31 \times 10^9$)	**0.001**	**0.012**	**0.013**
Lactobacillus rhamnosus	2.46×10^8 ($\pm 2.5 \times 10^8$)	2.6×10^8 ($\pm 9.11 \times 10^8$)	2.39×10^8 ($\pm 7.14 \times 10^8$)	0.062	0.07	0.752	9.25×10^7 ($\pm 1.48 \times 10^8$)	7.23×10^7 ($\pm 1.19 \times 10^8$)	0.064	0.07	0.649
Serratia marcescens	1.85×10^8 ($\pm 2.24 \times 10^8$)	4.61×10^7 ($\pm 1.18 \times 10^8$)	2.08×10^7 ($\pm 3.48 \times 10^7$)	**0.0002**	**0.0002**	0.629	3.49×10^8 ($\pm 1.01 \times 10^9$)	5.10×10^7 ($\pm 8.4 \times 10^7$)	0.424	0.424	0.196

* – p-value for the comparison of the number of bacteria in patients before treatment with healthy controls
** – p-value for the comparison of the number of bacteria in patients after treatment with healthy controls
*** – p-value for the comparison of the number of bacteria in patients before and after treatment
bold – statistically significant p value
SD – standard deviation

known that the balance between the Firmicutes, Gram-positive bacteria (e.g. depletion of Clades IV and XIVa Clostridia) and the Proteobacteria, Gram-negative bacteria (e.g. enrichment for the Enterobacteriaceae) and between Firmicutes and Gram-negative Bacteroidetes (including *Bacteroides*) is disturbed in CD. Different authors report both high and low Firmicutes/Bacteroidetes ratios [4,5,7,18]. These changes are also accompanied by a decrease in the biodiversity of intestinal microflora, which is observed in both luminal (fecal) and mucosal samples in patients with CD [7,17].

The role of Gram-negative anaerobic bacteria of the genus *Bacteroides* in the development of inflammation in the gastrointestinal tract is still investigated. Numerous studies indicate a decrease in the number of *Bacteroides,* including *B. fragilis,* in the course of CD [3,4], but there were also reports that those numbers are increasing [6,7]. Bacteria of the species *B. fragilis* produce polysaccharide A (PSA) which affects lymphocytes T_{Reg} producing anti-inflammatory cytokines (e.g. transforming growth factor beta - TGF-β or IL-10) which prevent the development of colitis [5]. However, Bloom et al. [19], demonstrated in a mouse model of IBD with human-relevant disease-susceptibility mutations, that commensal *Bacteroides* species could induce colitis only in certain genetic or environmental contexts. *B. fragilis*, being an enterotoxigenic strain (ETBF), may be associated with IBD. Remacle et al. [20], demonstrated that potential virulence factors of ETBF i.e., enterotoxin (fragilysin) and secretory metalloproteinase II (MPII), weaken cell-to-cell contacts and adherence junctions of intestinal mucosal barrier. Most of the observations discussed above are the result of experimental animal models. In our present study, the number of *B. fragilis* between the study groups did not differ significantly neither before nor after any type of treatment. Therefore, the relationship between this bacterium, CD and the treatment applied cannot be established on this basis. The observed positive correlation between the iron level and the number of *B. fragilis* is in line with numerous data indicating a strong dependence of this bacterium on heme, which in turn is conditioned by the iron level [21]. The presence of this correlation before and after the IFX treatment and the lack of significant correlations between iron levels and the number of *B. fragilis* in the EEN group allows us to conclude that the effect of the type of therapy on this correlation cannot be demonstrated.

S. marcescens is a Gram-negative bacterium belonging to the family Enterobacteriaceae, is commonly found in the environment and is also a possible source of nosocomial infections [12,22]. Its role in CD pathogenesis is unclear. Using next-generation sequencing, Hoarau et al. [23] have shown a significant increase in the amount of *S. marcescens* in CD patients. In our study, we found the opposite relationship (i.e. significantly smaller number of *S. marcescens* in the EEN group in comparison to the control group) but observed only in the EEN group. Perhaps this difference is related to the type of population studied (pediatric group with newly diagnosed CD and without the possibility of colonization of the gastrointestinal tract with bacteria from the hospital environment) or the implemented method to assess the bacterial count. To our knowledge, there is only one more study that indicates the association of *S. marcescens* with CD and further research is needed.

Many studies on gut microbiota in CD have focused on the Gram-positive Firmicutes bacteria of the genus *Lactobacillus*. Some authors reported a decrease while others observed an increase in the numbers of these microorganisms in the course of CD [17,18,24]. It may result from the fact that the genus *Lactobacillus* creates large groups of lactic acid bacteria (LAB), having more than 100 species. They are common in the environment and can sometimes have ambiguous effects on human health. LAB are also a part of oral and gastrointestinal microbiome, especially in breastfed infants. Certain species have probiotic properties [6,7,10,25]. In our study, we found no significant differences in the numbers of *L. rhamnosus* between the studied groups and the control group, which would suggest no direct influence of this bacterium on the course of CD. However, our analysis concerned *L. rhamnosus* globally as

a natural composition of the gut microbiota, while in many studies, the properties of this bacterium, as a specific, selected probiotic strain, have been evaluated for the use in the treatment of CD. There are many reports about the antimicrobial and anti-inflammatory effect of these bacteria, but there are also studies presenting inconclusive results [7,10,18]. However, our study focused primarily on the composition of the microbiome in health and disease, and did not analyze the therapeutic properties of individual species.

We also evaluated the second *Lactobacillus* species, which is considered to be a probiotic - *L. fermentum* - in the context of its contribution to the development and course of CD [10,26]. To our knowledge, this is the first such analysis. It is interesting, however, that in the EEN group the number of *L. fermentum* slightly increased during the treatment, whereas in the IFX group, it significantly decreased. Several factors may lead to such results, including the initial pretreatment status of children in each group, previous antibiotic therapy, and finally the treatment mode [2,3]. A larger number of *L. fermentum* in patients may suggest its involvement in the inflammatory process. Anderson et al. [27], have shown the role of the human oral isolate *L. fermentum* AGR1487 in the development of colitis in germ-free rats. Significantly higher numbers of *L. fermentum* in the IFX group than in the EEN group before treatment, as well as a significant decrease in the number of these bacteria in the IFX group after treatment and a decrease in disease activity expressed by a significant decrease in the PCDAI seem to confirm the hypothesis of proinflammatory activity of these bacteria. On the contrary, there are numerous studies indicating protective and immunomodulatory properties of *L. fermentum* in relation to colon mucosa [26,28]. This in turn is in line with high numbers of this species in the EEN group after treatment. We can speculate that a specific type of diet can contribute to this change [2,8]. However, the lack of precise determination of strains of this species of bacteria and their participation in the intestinal microbiota in our patients does not allow us to unequivocally explain the phenomenon described. Our results also suggest that bacterial strains of the same species can probably cause different host responses and highlight the importance of very precise strain characterization when considering the use of bacteria as probiotics in CD therapy.

4.1. Limitations of the study

As a limitation of our study we can mention that we have based only on fecal analysis, although we are aware that microbiome analysis in the tissue samples taken during biopsy would be a valuable addition to the study. However, to collect the biopsy, one needs to perform a colonoscopy. It is an invasive procedure and in children the examination is usually performed under general anesthesia. Also, the standards of clinical care do not include routine colonoscopy after the nutritional treatment or IFX induction therapy. Similarly, due to the lack of possibility of microbiological analysis of biopsy, we decided not to assess the involvement of *Escherichia coli* (especially the mucosal-bound adherent-invasive strain - AIEC). The role of this pathogen in IBD has been previously described in numerous studies [29,30], so we decided to choose those species of Gram-negative bacteria the role of which in this disease is unclear. The study covered selected bacterial species that were not representative of the whole microbiome. A comprehensive picture of changes in the microbiome could give the use of next-generation sequencing, but such analysis would only allow us to obtain percentage bacteria content, not absolute values as in our work.

5. Conclusions

Our observations are consistent with those of other authors and indicate that the composition of the microbiome varies between CD patients and healthy children. What is more, the implemented treatment (EEN or IFX) modifies the microbiome in patients, but does not make it become the same as in healthy controls. An increase in the number of *L. fermentum* (which is considered to be a probiotic) in patients on EEN may suggest a link between this bacteria and the diet used. Adverse changes in the number of *L. fermentum* in patients treated with IFX are likely to be the result of the longer duration of the disease and the previously used treatment.

The lack of unequivocal changes in the intestinal microbiome in the examined group of patients may also result from short observation time.

We can speculate that the selected bacteria examined by us may contribute to the development of the disease process in CD. However, these findings prompt for further research with more precise tools that would allow not only for identification of bacterial strains, but also for determination of their role in Crohn's disease.

Financial disclosure

The study was supported by Jagiellonian University Medical College in Krakow (Poland) within the framework of project grant no. K/ZDS/007042.

The author contribution

Study Design: Dominika Salamon, Tomasz Gosiewski, Kinga Kowalska-Duplaga, Krzysztof Fyderek.

Data Collection: Dominika Salamon, Kinga Kowalska-Duplaga, Agnieszka Krawczyk, Agnieszka Sroka-Oleksiak.

Statistical Analysis: Mariusz Duplaga.

Data Interpretation: Dominika Salamon, Tomasz Gosiewski, Kinga Kowalska-Duplaga, Krzysztof Fyderek, Mariusz Duplaga.

Manuscript Preparation: Dominika Salamon, Tomasz Gosiewski, Kinga Kowalska-Duplaga.

Literature Search: Dominika Salamon, Tomasz Gosiewski, Kinga Kowalska-Duplaga, Krzysztof Fyderek.

Funds Collection: Dominika Salamon, Tomasz Gosiewski.

Declaration of competing interest

The authors declare no conflict of interests.

References

[1] Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet 2017;390:2769–78.
[2] Uranga JA, López-Miranda V, Lombó F, Abalo R. Food, nutrients and nutraceuticals affecting the course of inflammatory bowel disease. Pharmacol Rep 2016;68:816–26.
[3] Lewis JD, Chen EZ, Baldassano RN, Otley AR, Griffiths AM, Lee D, et al. Inflammation, antibiotics, and diet as environmental stressors of the gut microbiome in pediatric Crohn's disease. Cell Host Microbe 2015;18:489–500.
[4] Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, et al. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 2006;55:205–11.
[5] Huttenhower C, Kostic AD, Xavier RJ. Inflammatory bowel disease as a model for translating the microbiome. Immunity 2014;40:843–54.
[6] Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. Gut 2016;65:330–9.
[7] McIlroy J, Ianiro G, Mukhopadhya I, Hansen R, Hold GL. Review article: the gut microbiome in inflammatory bowel disease—avenues for microbial management. Aliment Pharmacol Ther 2018;47:26–42.
[8] Ruemmele FM, Veres G, Kolho KL, Griffiths A, Levine A, Escher JC, et al. Consensus guidelines of ECCO/ESPGHAN on the medical management of pediatric Crohn's disease. J Crohn's Colitis 2014;8:1179–207.
[9] Hyams J, Crandall W, Kugathasan S, Griffiths A, Olson A, Johanns J, et al. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. Gastroenterology 2007;132:863–73.
[10] Alagón Fernández del Campo P, De Orta Pando A, Straface JI, López Vega JR, Toledo Plata D, Niezen Lugo SF, et al. The use of probiotic therapy to modulate the gut microbiota and dendritic cell responses in inflammatory bowel diseases. Med Sci 2019;7:33.
[11] Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, et al. Linking the human gut microbiome to inflammatory cytokine production capacity. Cell 2016;167:1125–1136.e8.
[12] Ochieng JB, Boisen N, Lindsay B, Santiago A, Ouma C, Ombok M, et al. Serratia

marcescens is injurious to intestinal epithelial cells. Gut Microb 2015;5:729–36.
[13] Levine A, Koletzko S, Turner D, Escher JC, Cucchiara S, De Ridder L, et al. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. J Pediatr Gastroenterol Nutr 2014;58:795–806.
[14] Kowalska-Duplaga K, Krawczyk A, Sroka-Oleksiak A, Salamon D, Wedrychowicz A, Fyderek K, et al. Dependence of colonization of the large intestine by candida on the treatment of Crohn's disease. Pol J Microbiol 2019;68:121–6.
[15] Gosiewski T, Szała L, Pietrzyk A, Brzychczy-Włoch M, Heczko PB, Bulanda M. Comparison of methods for isolation of bacterial and fungal DNA from human blood. Curr Microbiol 2014;68(2):149–155. https://doi.org/10.1007/s00284-013-0451-1.
[16] Bellaguarda E, Chang EB. IBD and the gut microbiota—from bench to personalized medicine. Curr Gastroenterol Rep 2015;17(4). https://doi.org/10.1007/s11894-015-0439-z.
[17] Fyderek K, Strus M, Kowalska-Duplaga K, Gosiewski T, Wędrychowicz A, Jedynak-Wąsowicz U, et al. Mucosal bacterial microflora and mucus layer thickness in adolescents with inflammatory bowel disease. World J Gastroenterol 2009;15:5287–94.
[18] Rodiño-Janeiro BK, Vicario M, Alonso-Cotoner C, Pascua-García R, Santos J. A review of microbiota and irritable bowel syndrome: future in therapies. Adv Ther 2018;35:289–310.
[19] Bloom SM, Bijanki VN, Nava GM, Sun L, Malvin NP, Donermeyer DL, et al. Commensal Bacteroides species induce colitis in host-genotype-specific fashion in a mouse model of inflammatory bowel disease. Cell Host Microbe 2011;9:390–403.
[20] Remacle AG, Shiryaev SA, Strongin AY. Distinct interactions with cellular e-cadherin of the two virulent metalloproteinases encoded by a bacteroides fragilis pathogenicity island. PloS One 2014;9:1–7.
[21] Yilmaz B, Li H. Gut microbiota and iron: the crucial actors in health and disease. Pharmaceuticals 2018;11:1–20.
[22] Joyner J, Wanless D, Sinigalliano CD, Lipp EK. Use of quantitative real-time PCR for direct detection of Serratia marcescens in marine and other aquatic environments. Appl Environ Microbiol 2014;80:1679–83.
[23] Hoarau G, Mukherjee PK, Gower-Rousseau C, Hager C, Chandra J, Retuerto MA, et al. Bacteriome and mycobiome interactions underscore microbial dysbiosis in familial Crohn's disease. mBio 2016;7:1–11.
[24] Wang W, Chen L, Zhou R, Wang X, Song L, Huang S, et al. Increased proportions of Bifidobacterium and the Lactobacillus group and loss of butyrate-producing bacteria in inflammatory bowel disease. J Clin Microbiol 2014;52:398–406.
[25] ŠtŠepetova J, Sepp E, Kolk H, Lõivukene K, Songisepp E, Mikelsaar M. Diversity and metabolic impact of intestinal Lactobacillus species in healthy adults and the elderly. Br J Nutr 2011;105:1235–44.
[26] Zhao Y, Hong K, Zhao J, Zhang H, Zhai Q, Chen W. Lactobacillus fermentum and its potential immunomodulatory properties. J Funct Foods 2019;56:21–32.
[27] Anderson RC, Ulluwishewa D, Young W, Ryan LJ, Henderson G, Meijerink M, et al. Human oral isolate Lactobacillus fermentum AGR1487 induces a pro-inflammatory response in germ-free rat colons. Sci Rep 2016;6:1–11.
[28] Pérez-Cano FJ, Dong H, Yaqoob P. In vitro immunomodulatory activity of Lactobacillus fermentum CECT5716 and Lactobacillus salivarius CECT5713: two probiotic strains isolated from human breast milk. Immunobiology 2010;215:996–1004.
[29] Palmela C, Chevarin C, Xu Z, Torres J, Sevrin G, Hirten R, et al. Adherent-invasive *Escherichia coli* in inflammatory bowel disease. Gut 2018;67:574–87.
[30] Perna A, Hay E, Contieri M, De Luca A, Guerra G, Lucariello A. Adherent-invasive *Escherichia coli* (AIEC): cause or consequence of inflammation, dysbiosis, and rupture of cellular joints in patients with IBD? J Cell Physiol 2020;235:5041–9.